A sustainable biotechnological process for the efficient synthesis of kojibiose

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ABSTRACT

This work reports the optimization of a cost-effective and scalable process for the enzymatic synthesis of kojibiose (2-*O*-α-D-glucopyranosyl-α-D-glucose) from readily available and low-cost substrates such as sucrose and lactose. This biotechnological process is based on the dextransucrase-catalysed initial synthesis of a galactosyl-derivative of kojibiose (4-*O*-β-D-galactopyranosyl-kojibiose) followed by the removal of residual monosaccharides by using a *Saccharomyces cerevisiae* yeast treatment, and a thorough hydrolysis step with *Kluyveromyces lactis* β-galactosidase. Depending on the final purification stage, i.e. extension of the yeast treatment or use of preparative liquid chromatography, the purity of the produced kojibiose ranged from 65% to ≥ 99%, respectively. The moderately high-yield achieved (38%, in weight respect to the initial amount of lactose) using this affordable synthesis process could expand the potential applications of kojibiose according to the bioactive properties that have been associated to this disaccharide, so far limited by its low availability.

Keywords: kojibiose, dextransucrase, β-galactosidase, yeast, prebiotic properties, anti-viral activities.

1. Introduction

Kojibiose is a naturally occurring disaccharide comprised of two glucose moieties bound through an α-(1→2) linkage (2-*O*-α-D-glucopyranosyl-α-D-glucopyranose) that can be found in sake and koji (cooked rice and/or soy beans inoculated with a fermentation culture of *Aspergillus oryzae*) extracts,[1] beer,[2] honey,[3,4] and starch hydrolyzate.[5] However, kojibiose is present in those food products at low levels making difficult its isolation from natural sources at high scale.

Kojibiose has been reported to be an excellent candidate as a prebiotic ingredient according to its *in vitro* selectivity of microbial fermentation using individual bacteria from *Bifidobacterium*, *Lactobacillus* or *Eubacterium* genera,[6] as well as with cultures of mixed human fecal bacteria.[7] The promising prebiotic potential of kojibiose and derived-oligosaccharides is also supported by the high resistance of α-(1→2) linkages to *in vitro* and *in vivo* gastrointestinal digestion.[8,9] Indeed, new biotechnological and sustainable approaches to the large scale manufacture of 1-2-linked oligosaccharides have been demanded considering that kojibiose is available only in limited amounts.[7]

Kojibiose has also the ability to specifically inhibit the α-glucosidase I in different tissues and/or organisms, such as rat liver microsomes,[10] bovine mammary gland,[11] yeast microsomal preparation[12] and mung bean seedlings[13] either on soluble or protein-bound oligosaccharides. Interestingly, glucosidase inhibitors have been shown to display important anti-viral activities[14,15] and, in particular, glycosidase I inhibition compounds, such as kojibose, have been suggested to open up new perspectives for the development of novel drugs, especially of the pseudodisaccharide class, for the treatment of human immunodeficiency virus type 1 (HIV-1) infections.[16] Additionally, due to their glucosidase inhibition activity, these compounds have been disclosed for

limiting digestion of dietary carbohydrates by inhibition of intestinal α-glucosidases thereby providing a regimen for treating diabetes mellitus and obesity.[17,18]

The best-known method for preparation of kojibiose is the isolation from a partial acetolyzate of dextran from *Leuconostoc mesenteroides* NRRL B-1299 by using a mixture of acetic anhydride, glacial acetic acid and concentrated sulphuric acid, as well as other chemical reagents such as chloroform or sodium methoxide.[19] Other attempts for the synthesis of kojibiose were based on the partial enzymatic hydrolysis of trisaccharides formed by dextransucrase actions,[20,21] as well as on the use of α-glucosidase, [22] glucoamylase,[23] sucrose phosphorylase[24] and kojibiose phosphorylase[25] using β-D-glucose-1-phosphate or 1,6-anhidro-β-D-glucopyranose as substrates. Nevertheless, all these methods are considered to be tedious, very time-consuming, uneconomical, and are normally characterized by the formation of by-products and low production efficiency.[6] These facts would explain the restricted quantities of kojibiose commercially available and its high cost, which limit its use despite the potential applications described above.

In this regard, this work reports the development of a novel biotechnological, cost-effective and environmentally-friendly process for the enzymatic synthesis of kojibiose with relatively high yield and purity from readily available and inexpensive raw materials such as sucrose and lactose. This method could straightforwardly be scaled-up to produce kojibiose at industrial scale, which would allow expanding the potential uses based on its bioactive properties, even allowing the reuse of important food-related by-products, such as cheese whey permeate and beet or cane molasses.

2. Experimental

Chemicals, reagents, standards and enzymes

All used chemicals and reagents were of analytical grade, purchased from Sigma-Aldrich (St. Louis, MO, USA), VWR (Barcelona, Spain), and Merck (Darmstadt, Germany). Ultra-pure water quality (18.2 MΩcm) with 1–5 ppb total organic carbon (TOC) and <0.001 EU mL^{-1} pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica, MA, USA).

Carbohydrates (fructose, glucose, galactose, sucrose, leucrose and lactose) were all purchased from Sigma-Aldrich (St. Louis, MO, USA) except kojibiose that was purchased from Carbosynth (Berkshire, United Kingdom), and lactosucrose that was from Wako Pure Chemical Industries (Osaka, Japan).

Dextransucrase from *Leuconostoc mesenteroides* B-512F was purchased from CRITT Bio-Industries (Toulouse, France). Specific activity was 0.4 U mg^{-1}, where 1 unit is the amount of enzyme required to perform the transfer of 1 μmol of glucose per minute at a working temperature of 30 °C, a sucrose concentration of 100 g L^{-1} at pH 5.2 in 20 mM sodium acetate buffer with 0.34 mM of $CaCl_2$. Soluble commercial preparation of β-galactosidase from *Kluyveromyces lactis* (Lactozym Pure 6500 L) was kindly supplied by Novozymes (Bagsvaerd, Denmark).

Steps of the process for the synthesis of kojibiose

Enzymatic synthesis of 4'-galactosyl-kojibiose

The synthesis of the trisaccharide 4'-galactosyl-kojibiose, also denominated 2-α-glucosyl-lactose (O-β-D-galactopyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-α-D-glucopyranose) was carried out by transglucosylation reaction catalyzed by dextransucrase from *L. mesenteroides* B-512F (0.8 U mL^{-1}) at 30 ºC in 20 mM sodium acetate buffer with 0.34 mM $CaCl_2$ (pH 5.2) in the presence of sucrose (donor) and

lactose (acceptor) as described previously by Díez-Municio et al.[26] at two different concentration ratios (25:25 or 10:25, expressed in g/100 mL). The reaction proceeded for 24 h after which the enzyme was inactivated by heating at 100 ºC for 5 min.

Yeast treatment with Saccharomyces cerevisiae

Removal of the monosaccharides (fructose, glucose, galactose) and sucrose present in the carbohydrate mixture was carried out by yeast treatment. Since it is well-known that one of the main affecting factors for removal of carbohydrates by yeast is the initial sugar concentration, this value was fixed at 200 g L^{-1}.[27] Treatment with *Saccharomyces cerevisiae* fresh baker's yeast (Levital, Panibérica de Levadura, Valladolid, Spain) took place at 30 ºC under stirring (1,200 rpm), with the addition of 16 g of yeast per 100 g of sugar. When needed, the yeast was removed by centrifugation (5 min at 8,000 rpm) or filtration.

β-galactosidase hydrolysis

The hydrolysis of the remaining lactose and trisaccharide 4'-galactosyl-kojibiose was carried out by adding β-galactosidase enzyme from *K. lactis* (Lactozym Pure 6500 L) (65 U mL^{-1}) to the reaction medium without removing the yeast cells. The temperature of the reaction was maintained at 30 ºC. Prior to the addition of the β-galactosidase enzyme, the pH was regulated at 7.3 using potassium hydroxide (5 M), and magnesium chloride (5 mM) was added. The hydrolysis reaction was performed for 90 min, after which the enzyme was inactivated by the pH drop caused by the still ongoing yeast treatment.

Purification of kojibiose by preparative liquid chromatography

Kojibiose was isolated and purified by liquid chromatography with refractive index detector (LC-RID) from the reaction mixture obtained after the treatment by β-galactosidase from *K. lactis* on an Agilent Technologies 1260 Infinity LC System

(Boeblingen, Germany) using a Zorbax NH_2 PrepHT preparative column (250 × 21.2 mm, 7 µm particle size) (Agilent Technologies, Madrid, Spain). Two mL of reaction mixtures (150 mg of total carbohydrates) were eluted with acetonitrile:water (75:25, *v/v*) as the mobile phase at a flow rate of 21.0 mL min^{-1} for 30 min. The separated kojibiose was collected using an Agilent Technologies 1260 Infinity preparative-scale fraction collector (Boeblingen, Germany), and the fractions were pooled, evaporated in a rotatory evaporator R-200 (Büchi Labortechnik AG, Flawil, Switzerland) below 25 ºC and freeze-dried.

Analytical techniques

Liquid chromatography with refractive index detector (LC-RID).

The progress of the kojibiose synthesis process was monitored by liquid chromatography with refractive index detector (LC-RID) on an Agilent Technologies 1220 Infinity LC System – 1260 RID (Boeblingen, Germany). The separation of carbohydrates was carried out with a Kromasil (100-NH_2) column (250 × 4.6 mm, 5 µm particle size) (Akzo Nobel, Brewster, NY, USA) using isocratic elution with acetonitrile:water (75:25, *v/v*) as the mobile phase at a flow rate of 1.0 mL min^{-1} for 40 min. Injection volume was 50 µL (1 mg of total carbohydrates). Data acquisition and processing were performed using Agilent ChemStation software (Agilent Technologies, Boeblingen, Germany).

Carbohydrates in the reaction mixtures were identified by comparing their retention times with those of standard sugars. Quantitative analysis was performed by the external standard method, using calibration curves in the range 0.1-10 mg mL^{-1} for fructose (quantification of monosaccharides), sucrose, leucrose, lactose, kojibiose and 4'-galactosyl-kojibiose. All analyses were carried out in triplicate. Determination

coefficients obtained from these calibration curves, which were linear over the range studied, were high ($R^2 > 0.999$). Reproducibility of the method was estimated on the basis of the intra-day and inter-day precision, calculated as the relative standard deviation (*RSD*) of concentrations of oligosaccharide standards obtained in $n \geq 5$ independent measurements, obtaining *RSD* values below 10% in all cases.

Gas chromatography with a flame ionization detector (GC-FID)

The carbohydrate composition of the resulting reaction mixture was determined by GC-FID on an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector, using nitrogen as carrier gas at 1 mL min^{-1}. The trimethylsilyl oxime (TMSO) derivatives were prepared as previously described by Sanz et al.[28] and separated using an fused-silica capillary column (30 m × 0.32 mm i.d. × 0.5 μm film thickness) SPBTM–17, bonded, crosslinked phase (50% diphenyl / 50% dimethylsiloxane) (Supelco, Bellefonte, PA, USA). The oven initial temperature was 200 ºC, increased at a rate of 4 ºC min^{-1} to 230 ºC, then at a rate of 2 ºC min^{-1} to 290 ºC and held for 50 min. The injector and detector temperatures were 280 and 290 ºC, respectively. Injections were made in the split mode (1:20). Data acquisition and integration were performed using Agilent ChemStation software (Wilmington, DE, USA). Quantitative data for carbohydrates were calculated from FID peak areas relative to phenyl-β-D-glucoside (internal standard). Mixtures of standard solutions of fructose, galactose, glucose, sucrose, lactose, leucrose, kojibiose and lactosucrose over the expected concentration range were prepared with 0.2 mg of internal standard to calculate the response factor for each sugar.

Gas chromatography with mass spectrometry detection (GC-MS)

Both synthesized and commercial kojibiose were analyzed by GC-MS on an Agilent Technologies 7890A gas chromatograph coupled to a 5975C MSD quadrupole mass detector (Agilent Technologies, Wilmington, DE, USA) in order to confirm the identification of the purified kojibiose. Sugars separation was performed under the same chromatographic conditions described above, substituting the carrier gas with helium. The mass spectrometry detector was operated in electronic impact mode at 70 eV. Mass spectra were acquired using Agilent ChemStation MSD software (Wilmington, DE, USA).

Chemical and microbiological characterization of the synthesized kojibiose

Chemical analyses of kojibiose

Chemical quality parameters (dry matter, mineral composition, nitrogen and pH) were determined in kojibiose samples.

The dry matter (DM) content was gravimetrically determined by drying the kojibiose samples in a conventional oven at 102 ºC until constant weight, according to the AOAC method.[29]

Ion composition of the kojibiose samples was determined using an ICP-MS NexION 300XX Perkin Elmer instrument (Perkin Elmer, Waltham, MA, USA). Either a semiquantitative analysis or a quantitative analysis of the elements of interest using the external calibration method and internal standards to correct instrumental drift were carried out.[30] Nitrogen percentage determination was performed on an elemental analyzer LECO CHNS-932 (LECO Corporation, St. Joseph, MI, USA).

The pH of kojibiose samples was measured using a pH meter (MP 230, Mettler-Toledo, Barcelona, Spain) at a concentration of 10 mg mL^{-1}.

Microbiological analysis of kojibiose

In order to evaluate the microbiological quality, samples were analyzed for the presence of yeasts and molds, total and sporulated aerobic microorganisms and enterobacteria. Serial dilutions were performed in triplicate with peptone water (Biocult BV, Roelofarendsveen, The Netherlands). Yeasts and molds were plated on Sabouraud chloramphenicol agar and incubated at 25 ± 1 °C for 5 days. The total and sporulated aerobic bacteria were determined by plating appropriately diluted samples onto plate count agar. The samples were incubated at 30 ± 1 °C for 72 h for total aerobic bacteria and at 37 ± 1 °C for 48 h for sporulated aerobic bacteria after heat treatment of stock dilution at 80 °C for 10 min. For enterobacteria counts, violet red bile dextrose agar was used and incubation was carried out at 30 ± 1 °C for 24 h. All microbial counts were reported as colony forming units per gram (cfu g^{-1}). All culture media were of Difco (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

3. Results and Discussion

Optimization of the process for the efficient synthesis of kojibiose

A general scheme illustrating the process followed for the synthesis of kojibiose is shown in **Figure 1**. Briefly, this procedure, which uses cheap food-grade enzymes and starting substrates as sucrose and lactose, does not generate any toxic substances and is comprised of four steps carried out at constant temperature (30 ºC): i) synthesis of a galactosyl-derivative of kojibiose catalyzed by *Leuconostoc mesenteroides* B-512F dextransucrase; ii) removal of remaining monosaccharides and sucrose by *Saccharomyces cerevisiae* yeast; iii) production of kojibiose by the hydrolytic action of β-galactosidase from *Kluyveromyces lactis*, and; iv) purification stage based on the initial treatment with yeast or on a preparative liquid chromatographic separation. This

process was optimized to maximize kojibiose yield and purity, as well as to reduce operating time as it is explained below.

1st step: Enzymatic synthesis of the trisaccharide 4'-galactosyl-kojibiose

Based on a previous work dealing with the optimization of the enzymatic synthesis of the trisaccharide *O*-β-D-galactopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-(1→2)]-α-D-glucopyranose,[26] the concentration of the initial substrates (sucrose and lactose) was set at 25% *(w/v)* each, whereas the enzyme concentration employed (i.e., dextransucrase from *L. mesenteroides* B-512F) was 0.8 U mL^{-1} and the reaction time 24 h. This trisaccharide was formed by the dextransucrase-catalysed transfer of a glucosyl unit from the hydrolysis of sucrose to lactose acceptor through the formation of an α-(1→2)-glucosyl bond (**Figure 2**). Under these optimized conditions, the final reaction mixture was composed of 31.2% 4'-galactosyl-kojibiose and 38.5% lactose, 21.3% fructose, 5.2% leucrose, 2.8% lactosucrose, 0.9% glucose and 0.1% sucrose as determined by LC-RID (**Table 1**).

2nd step: Yeast treatment with Saccharomyces cerevisiae

Monosaccharides such as glucose, galactose and fructose are potent inhibitors of the hydrolytic action of β-galactosidase.[31] Considering the high levels of fructose present in the reaction mixture, its removal was required prior to the hydrolysis step with the β-galactosidase. Besides, its removal would also help to obtain a more purified kojibiose. *S. cerevisiae* yeast treatments have been already successfully used to remove monosaccharides (mainly glucose and galactose) from galacto-oligosaccharides mixtures.[32-34] Yoon et al.[35] also demonstrated that yeast cells are very efficient for the removal of fructose and sucrose whilst lactose and other oligosaccharides formed by transglycosylation or condensation reactions remained unaffected. Apart from the high efficiency of yeast treatment in the removal of monosaccharides, this procedure can also

249 be performed directly on synthesis mixtures without the need of significant dilutions (as
250 required by other techniques such as nanofiltration) and is also a low-cost and easily
251 scalable process for industrial uses.[32,36]

252 In order to reduce the treatment time, two different yeast charges were assayed
253 (16 and 32 mg mL^{-1}) and the process was left to stand for 7 h. The carbohydrate
254 composition of the mixture was characterized and quantified by LC-RID (data not
255 shown) and the pH was monitored every 30 min (**Figure 3**). With both yeast charges the
256 pH decreased from 5.2 to 3.7 as sugars were metabolized, and this pH decrease could be
257 caused by the dissolution of CO_2 (produced during glycolysis) resulting in carbonic
258 acid. This treatment was carried out under aeration and vigorous stirring to facilitate the
259 yeast growth and evaporation of ethanol.

260 In the case of the highest yeast charge, a pH plateau was achieved after 4 h of
261 treatment (**Figure 3**). This decrease is in agreement with the efficient decrease in
262 fructose (94.4% of fructose was removed) and the complete removal of lactosucrose,
263 glucose and sucrose observed (**Table 1**). In contrast, leucrose, lactose and 4'-galactosyl-
264 kojibiose were unaffected after the whole yeast treatment. In consequence, a yeast
265 charge of 32 mg mL^{-1} and 4 h were the parameters established for this second stage.

266 *3rd step: Hydrolysis with β-galactosidase from Kluyveromyces lactis*

267 This stage started when the monosaccharides were almost completely removed,
268 although the yeast cells employed were still maintained in the reaction medium. Due to
269 the optimum pH for the hydrolytic action of β-galactosidase from *K. lactis* is in the
270 range 6.5-7.5,[37] after the yeast treatment the pH was increased to 7.3 and three different
271 enzyme concentrations, namely, 6.5, 32.5 and 65 U mL^{-1}, were assayed. As indicated
272 above, the carbohydrate composition of the mixture was quantified by LC-RID and the
273 pH was monitored at different times (data not shown). Under the three enzyme

concentrations studied, the trisaccharide 4'-galactosyl-kojibiose and lactose were efficiently hydrolyzed to release kojibiose and galactose, as well as glucose and galactose, respectively (**Figure 2**). Moreover, no detectable formation of galacto-oligosaccharides derived from the transgalactosylation of lactose was observed. With the highest β-galactosidase concentration assayed, the hydrolysis was faster and after 90 min no trace of lactose was detected and the 4'-galactosyl-kojibiose trisaccharide was completely hydrolyzed. Besides, part of the monosaccharides produced during the enzymatic hydrolysis was consumed by the yeast present in the medium producing a pH drop down to 6.0 and, consequently, the enzyme was inactivated. Under these conditions, the reaction mixture after this hydrolysis step was comprised of 46.5% kojibiose, 20.2% glucose, 19.0% galactose, 11.3% leucrose, 2.9% unidentified trisaccharides, and 0.1% fructose (**Table 1**).

4th step: Purification of kojibiose

For the final step, two different strategies were studied as it is shown in **Figure 1**.

i) *Yeast treatment purification*

This strategy consists in maintaining the yeast treatment for additional 42 h, thanks to the yeast already present in the reaction medium. This second yeast treatment took longer since, at this point, it was necessary to eliminate both glucose and galactose; during the initial 3 h the yeast mainly metabolized glucose while galactose levels remained constant and, once glucose was removed, galactose assimilation was observed (data not shown) at a slower rate. The main advantage of this step relies on that no additional charge of yeast is required and, consequently, the yeast concentration added during the second step was enough to eliminate the monosaccharide fraction. Nevertheless, other attempts based on the addition of a second charge of yeast during

this step were carried out, although these modifications did not lead to shorten the incubation time (data not shown).

The final carbohydrate composition after the incubation with the yeast was as follows: 73.9% kojibiose, 18.4% leucrose and 7.7% unidentified trisaccharides (**Table 1**). **Figure 4B** displays a GC-FID chromatogram of the final sample and the **Figure 5B** shows the corresponding mass spectrum which allowed the kojibiose identification by comparison with the respective GC retention index and MS data of the commercial standard (**Figures 4A** and **5A**). The high abundance of *m/z* 319 ion (ratio 319/361 > 1) is characteristic of aldohexoses having a 1→2 glycosidic linkage originated by the loss of a *O*-trimethylsilyl (TMS)OH group from the chain C3–C4–C5–C6, as has been previously described.[38,39]

Since leucrose was neither metabolized by the yeast treatment nor hydrolyzed by the β-galactosidase and given that its formation proves that fructose can also act as a minor acceptor in the dextransucrase-catalyzed reactions,[40] additional synthesis studies starting with a low concentration of sucrose (10%, *w/v*) and keeping the lactose concentration at 25% *(w/v)* were performed in order to reduce the final content of leucrose. This was attained although substantial levels of di- and trisaccharides derived from the transgalactosylation of lactose were also found and, consequently, the purity of kojibiose could not be increased (**Table 2**). The formation of galacto-oligosaccharides was probably favored because a higher concentration of lactose remained after formation of the 4'-galactosyl-kojibiose, making easier its transgalactosylation.

ii) *Chromatographic purification*

A second strategy based on the purification by preparative liquid chromatography with refractive index detector (LC-RID) was attempted with the aim of increasing the purity of kojibiose and reducing the total process time. In this case, the

first step was to remove from the medium the remaining yeast either by centrifugation or by filtration. As it is shown in **Figure 6**, kojibiose was well resolved from the rest of carbohydrates present, including disaccharides as leucrose, within only 30 min. This purification step allowed the attainment of kojibiose at the gram scale. Finally, the purity grade of the chromatographically isolated kojibiose was checked by GC-FID (**Figure 4C**), this being comparable to that of the commercial standard which is labeled as min. 99% (**Figure 4A**).

Physico-chemical and microbiological characterization of the synthesized kojibiose

Apart from the characterization of the carbohydrate composition, the synthesized kojibiose was also subjected to a physico-chemical and microbiological characterization (**Table 2**). Both the kojibiose purified by the continuous yeast treatment or chromatographically isolated were freeze-dried and white powders were obtained. However, the latter required an evaporation step before freeze-drying to remove the acetonitrile present in the mobile phase. Likewise, the estimated yield of kojibiose synthesized with the process described in this work was 38% (in weight respect to the initial amount of lactose).

Additionally, analyses of the dry matter, nitrogen, mineral composition, and pH were carried out for the kojibiose purified with the yeast treatment. Whilst the purity of kojibiose isolated on the preparative LC column could be considered ≥ 99% as it was indicated above, the content of kojibiose purified by the yeast treatment was 65% on dry matter. The second most important compound was leucrose (19%) followed by 8% of yeast metabolites produced when sugars are metabolized (mainly minor amounts of polyalcohols and/or organic acids) (**Table 2**). Lastly, microbiological assays demonstrated that the microbial load (yeast and molds, total and sporulated aerobic

bacteria, enterobacteria) was, in all cases, lower than 3×10^1 cfu g^{-1}, indicating that the kojibiose synthesized by this process is microbiologically safe and could be used as food ingredient, among other applications. Lastly, this procedure, especially when the purification is carried out with yeast treatment, can be considered as an environmental friendly process since the use of toxic solvents is avoided during the whole treatment.

4. Conclusions

This work describes a cost-effective and scalable process developed at a moderate temperature, 30 ºC, for the efficient synthesis of kojibiose from abundant and low-cost substrates as sucrose and lactose. This biotechnological process could be an alternative to the chemistry-based procedures used for the production of kojibiose, as well as to be further applied to important agro-industrial residues containing sucrose and lactose, such as beet and cane molasses or cheese whey permeate.[41] The reasonably high-yield and affordable synthesis of such a value-added ingredient, as kojibiose, from food-related by-products provides new opportunities for potential applications of kojibiose considering its limited availability, as well as for the valorization of by-products from the sugar and dairy industries.

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Figure legends

Figure 1. Biotechnological process scheme implemented for the efficient synthesis of kojibiose by transglucosylation of lactose catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F, yeast treatment with *Saccharomyces cerevisiae* and hydrolysis with β-galactosidase from *Kluyveromyces lactis* with and without subsequent chromatographic purification.

Figure 2. Chemical structures of the carbohydrates involved in the developed kojibiose synthesis process.

Figure 3. Representative time course showing the different pH values achieved during the treatment with *Saccharomyces cerevisiae* at two different yeast charges (16 and 32 mg mL^{-1}). The carbohydrate mixture (produced in the dextransucrase-catalysed reaction) used for the yeast treatment had a 200 mg mL^{-1} total sugar concentration.

Figure 4. Profiles obtained by gas chromatography with a flame ionization detector (GC-FID) of the commercial kojibiose standard (A) and the synthesized kojibiose obtained with yeast (B) or chromatographic (C) purification.
Peak identification: 1 and 2 (yeast metabolites), 3 (internal standard), 4 and 5 (leucrose), 6 and 7 (kojibiose), 8 (trisaccharides).

Figure 5. Mass spectra obtained by gas chromatography coupled to mass spectrometry (GC-MS) analysis using the corresponding trimethylsilyl oximes (TMSO) of the commercial kojibiose standard (A) and the synthesized kojibiose obtained with yeast purification (B).

Figure 6. Preparative liquid chromatography with refractive index detector (LC-RID) profile obtained in the chromatographic purification step.

471 **Table 1.** Carbohydrate composition (mg mL^{-1}) of the different mixtures determined by
472 LC-RID during the four steps involved in the process for the efficient synthesis of
473 kojibiose. The concentration of the initial substrates (sucrose and lactose) was set at
474 25% *(w/v)* each (500 mg mL^{-1} total sugar concentration).

	1st step	2nd step	3rd step	4th step	
	Dextransucrase Synthesis	**Yeast Treatment**	**β-galactosidase Hydrolysis**	**Yeast Treatment**	**LC-RID Purification**
Fructose	99.55 ± 3.40	5.55 ± 0.05	0.13 ± 0.06	-	-
Glucose	4.25 ± 0.29	-	41.34 ± 4.68	-	-
Galactose	-	-	38.81 ± 0.26	-	-
Sucrose	0.67 ± 0.01	-	-	-	-
Leucrose	24.22 ± 0.64	24.96 ± 0.11	23.18 ± 0.15	24.12 ± 0.29	-
Kojibiose	-	-	95.06 ± 1.84	96.58 ± 2.49	97.42 ± 8.38
Lactose	179.95 ± 5.53	193.09 ± 0.44	-	-	-
Lactosucrose	13.28 ± 0.38	-	-	-	-
4'-galactosyl-kojibiose	145.86 ± 5.61	150.04 ± 1.50	-	-	-
Unidentified trisaccharides	-	-	5.93 ± 0.08	10.05 ± 0.32	-

475

476

477 **Table 2.** Chemical and microbiological characterization of the product with high
478 content in kojibiose obtained by dextransucrase-catalysed reaction, subsequent
479 treatment with *Saccharomyces cerevisiae* yeast and β-galactosidase from
480 *Kluyveromyces lactis*, including a final yeast purification step.

481

	Suc 25% + Lac 25% †	Suc 10% + Lac 25% ‡
Chemical analysis		
Dry matter (DM)	93% on product	93% on product
Kojibiose§	65% on DM	52% on DM
Leucrose§	19% on DM	8% on DM
Disaccharides§ (galactosylated derivatives)	-	16% on DM
Trisaccharides§	6% on DM	14% on DM
Yeast metabolites§	8% on DM	8% on DM
Minerals	2% on DM	2% on DM
Potassium	1.40%	1.40%
Magnesium	0.20%	0.20%
Nitrogen	0.10%	0.10%
Sodium	0.10%	0.10%
Others	0.10%	0.10%
pH ¶	5.0	5.0
Microbiological analysis		
Yeasts and molds	$< 3 \times 10^1$ cfu g^{-1}	$< 3 \times 10^1$ cfu g^{-1}
Total viable aerobic at 30 °C	$< 3 \times 10^1$ cfu g^{-1}	$< 3 \times 10^1$ cfu g^{-1}
Aerobic sporulated at 30 °C	$< 1 \times 10^1$ cfu g^{-1}	$< 1 \times 10^1$ cfu g^{-1}
Enterobacteriaceae	$< 1 \times 10^1$ cfu g^{-1}	$< 1 \times 10^1$ cfu g^{-1}

482 †The concentration of the initial substrates, sucrose (Suc) and lactose (Lac)
483 was set at 25% *(w/v)* each (500 mg mL^{-1} total sugar concentration).

484 ‡The concentration of the initial sucrose (Suc) was 10% *(w/v)* and lactose
485 (Lac) 25% *(w/v)* (350 mg mL^{-1} total sugar concentration).

486 §Determined by GC-FID.

487 ¶Values obtained by measuring the pH of a solution of 10 mg of product
488 dissolved in 1 ml of ultrapure water.

489